A Rapid Microbioassay for Discovery of Novel Fungicides for *Phytophthora* spp.

Jeanne M. Kuhajek, Steven N. Jeffers, Marc Slattery, and David E. Wedge

First and third authors: Department of Pharmacognosy and The National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University 38677; second author: Department of Plant Pathology and Physiology, Clemson University, Clemson, SC 29734; and fourth author: U.S. Department of Agriculture-Agricultural Research Service, NPURU, The National Center for Natural Products Research, The University of Mississippi, University 38677.
Current address of J. M. Kuhajek: Landcare Research, P.O. Box 69, Lincoln 8152, New Zealand.
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ABSTRACT

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A microbioassay was developed for the discovery of compounds that inhibit *Phytophthora* spp. This assay uses a 96-well format for high-throughput capability and a standardized method for quantitation of initial zoospore concentrations for maximum reproducibility. Zoospore suspensions were quantifiable between 0.7 and 1.5×10^5 zoospores per ml using percent transmittance (620 nm). Subsequent growth of mycelia was monitored by measuring optical density (620 nm) at 24-h intervals for 96 h. Full- and half-strength preparations of each of three media (V8 broth, Roswell Park Memorial Institute mycological broth [RPMI], and mineral salts medium) and four zoospore concentrations (10, 100, 1,000, and 10,000 zoospores per ml) were evaluated. Both full- and half-strength RPMI were identified as suitable synthetic media for growing *P. nicotianae*, and 1,000 zoospores per ml was established as the optimum initial concentration. The assay was used to determine effective concentration values for 50% growth reduction (EC_{50}) for seven commercial antifungal compounds (azoxystrobin, fosetyl-aluminum, etridiazole, metalaxyl, pentachloronitrobenzene, pimaricin, and propamocarb). These EC_{50} values were compared with those obtained by measuring linear growth of mycelia on fungicide-amended medium. The microbioassay proved to be a rapid, reproducible, and efficient method for testing the efficacy of compounds that inhibit spore germination in *P. nicotianae* and should be effective for other species of *Phytophthora* as well. The assay requires relatively small amounts of a test compound and is suitable for the evaluation of natural product samples.

Several species in the genus *Phytophthora* cause diseases that result in devastating losses to a wide variety of plants. These diseases, including root and crown rots, cankers, foliar blights, and fruit rots, affect food and fiber crops, forest trees, and a variety of ornamental plants (1,20). The impact of *Phytophthora* spp. on food and fiber production is enormous (20,52). In the United States alone, crop losses attributed to these pathogens exceed several billion dollars annually (20). Worldwide, costs for chemical management of Phytophthora diseases represent over 25% of the annual fungicide market (47,48). Advances in cultural practices, disease forecasting programs, and the availability of resistant cultivars have improved disease management, but fungicides remain an indispensable component of any effective disease management program (20).

Chemicals effective against *Phytophthora* spp. are limited. Many broad-spectrum fungicides are not effective against these organisms (15) and few alternatives are available. The development of several chemicals specifically targeting *Phytophthora* spp. and other oomycetes (e.g., etridiazole, metalaxyl, mefenoxam, and fosetyl-Al) has improved management of Phytophthora diseases (1). However, the rapid regeneration times and exceptional adaptability of *Phytophthora* spp. (6) have resulted in the development of fungicide resistance within specific pathogen populations, e.g., *P. infestans* on potatoes (11,12,14), *P. capsici* on peppers and cucurbits (33,41,43), and *P. nicotianae* (=*P. parasitica*) and *P. citricola* on ornamental hosts (16,25). There is potential for development of resistance in other populations of *Phytophthora* spp. Given the enormous economic impact of Phytophthora diseases worldwide, discovery of new and novel chemicals to manage these pathogens is an important priority in fungicide research.

Discovery of new fungicides for the management of *Phytophthora* spp. relies primarily on in vivo assessment of efficacy on greenhouse-grown plants (L. D. Houseworth, Syngenta, *personal communication*). This method is time consuming, material and labor intensive, and requires relatively large quantities of test compounds. In vitro assays (e.g., growth or development of fungi on fungicide-amended agar medium) often supplement in vivo studies (7,23,24,37,46); however, these too are tedious and relatively large scale. Although these methods are adequate for evaluating compounds derived from synthetic sources, they are unsuitable for assessing natural products and other limited quantity samples.

Natural products are a prolific source of chemical diversity and provide enormous potential for the discovery of novel biologically active compounds (56). They remain virtually untapped for plant disease management, however, primarily because initial quantities of natural product extracts and test compounds often are insufficient for evaluation using traditional bioassay methods. A need exists for an effective and efficient method to screen natural product samples for potential inhibitory activity against *Phytophthora* spp. and other plant pathogens.

The purpose of this study was to develop a rapid, quantitative, microscale bioassay for the discovery of novel compounds with efficacy against *Phytophthora* spp. In vitro assessment of *Phytophthora* spp. presents a challenge because the diverse life stages of these organisms are differentially sensitive to the inhibitory effects of fungicides (7,23,24,37,46,53). Any single bioassay likely will miss some active compounds; however, development

Corresponding author: J. M. Kuhajek
E-mail address: kuhajekj@landcareresearch.co.nz

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of an efficient and reproducible method for the preliminary evaluation of natural products and other samples of limited quantity is needed. The specific aims of this study were to (i) standardize initial zoospore concentrations; (ii) identify a suitable synthetic medium for quantifiable growth; (iii) determine the optimum initial zoospore concentration for the selected medium; and (iv) compare the microbioassay with the standard fungicide-amended agar assay.

MATERIALS AND METHODS

Fungi. *P. nicotianae* Breda de Haan (=*P. parasitica* Dastur) was selected as a model species for assay development because it has a broad host range (20) and readily and consistently produces abundant quantities of zoospores in vitro. Isolates used in this study (AF016, D003, and D119) were recovered from diseased ornamental plants in South Carolina and are maintained by S. N. Jeffers in a permanent collection in the Department of Plant Pathology and Physiology, Clemson University, South Carolina. Axenic cultures of each isolate were grown in the dark at 26°C on V8-juice agar (V8A) (200 ml of V8 juice [Campbell Soup Co., Camden, NJ], 15 g of Difco Bacto agar [Difco Laboratories, Detroit], 3 g of $CaCO_3$, and 800 ml of distilled water). After 4 days, 5-mm agar plugs from the leading edge of individual colonies were transferred into several sterile 1.5-ml microcentrifuge tubes containing 1 ml of sterile distilled water, and tubes were stored in the dark at 15°C (2,4).

Zoospore production. Zoospores were produced under aseptic conditions following a modification of the procedure reported by Chen and Zentmyer (9). Plugs from storage tubes were transferred onto V8A and incubated in the dark at 26°C. Fifteen 5-mm agar plugs were transferred from the leading edge of a 4-day-old colony to a disposable petri dish (60 × 15 mm) containing 5 ml of sterile mineral salts solution (MSS): 3.08 g of $Ca(NO_3)_2{\cdot}4H_2O$, 1.49 g of $MgSO_4{\cdot}7H_2O$, and 0.51 g of KNO_3 in 1 liter of distilled water autoclaved for 15 min at 121°C and then supplemented with 1 ml of chelated iron solution (6.525 g of EDTA, 0.375 g of KOH, and 1.245 g of $FeSO_4{\cdot}7H_2O$ in 50 ml of distilled water and filter-sterilized through a 0.2-μm Millipore membrane filter). Dishes were placed under continuous light at 20°C (i.e., approximately 15 cm beneath four fluorescent tubes [17 W, cool white, 85 ± 5 μmol m^{-2} s^{-1}]) to induce sporangium production. At 24 h, the salt solution was replaced with fresh solution. After 48 h, the salt solution was removed and the plugs were rinsed three times with 5 ml of sterile distilled water and covered with 5 ml of the same. To induce synchronous cleavage and release of zoospores, dishes were placed at 4°C for 20 min and then moved back under lights at 20°C for 3 h. The zoospore suspension from each dish was transferred to a 15-ml conical tube and left undisturbed for 5 min to allow mycelium fragments and other heavy particles to settle. The upper 4 ml, containing free-swimming zoospores, was transferred to a second tube and vortexed for 70 s to encyst zoospores. Zoospores were stained with rose bengal (300 ppm in aqueous solution) to verify viability; the number of colorless (viable) zoospores was counted with a hemacytometer (26). Concentrations of viable zoospores typically were around 1×10^5 zoospores per ml.

Suspensions of encysted nonstained zoospores were adjusted to the linear range of transmittance with sterile distilled water and standardized to a specified zoospore concentration by dilution with medium. The linear range of transmittance was determined by plotting a regression line between percent transmittance and zoospore concentration. A stock zoospore suspension from isolate AF016 was diluted serially with sterile distilled water to yield 10 concentrations. After thorough mixing, each suspension was enumerated with a hemacytometer and evaluated for percent transmittance at 620 nm with a spectrophotometer (slit width of 3 nm; Shimadzu UV-3101PC; Shimadzu Scientific Instruments, Columbia, MD); counts and readings were conducted three times for each sample to yield a mean value. Means were pooled from three separate experiments repeated in time.

Evaluation of media. Growth of the three isolates of *P. nicotianae* was evaluated in full- and half-strength preparations of two synthetic and one natural liquid media. Synthetic media were Roswell Park Memorial Institute mycological broth (RPMI: 16.2 g of RPMI 1640 medium per liter, with L-glutamine and pH indicator and without bicarbonate [Life Technologies, Grand Island, NY]) and mineral salts medium (MSM: MSS amended with 20 g of D-glucose per liter and 0.8 mg of thiamin per liter). The natural medium was clarified V8 broth (V8B: 200 ml of clarified V8 juice [i.e., centrifuged at 10,000 rpm for 10 min and filtered through Miracloth] and 800 ml of deionized distilled water). All media were buffered with 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (34.5 g/liter; Sigma Chemical Co., St. Louis), adjusted to pH 7.0 ± 0.1 at 25°C with 1.0 N NaOH, and filter sterilized through a 0.2-μm Millipore membrane filter (38) to remove particulates that could interfere with photometric growth evaluation.

Each medium was dispensed into 14 replicate wells of a 96-well flat-bottom microtiter plate (Nunc MicroWell, untreated; Roskilde, Denmark). Each test well received 100 μl of medium and 100 μl of zoospore suspension at 1,000 zoospores per ml for a total well volume of 200 μl (5) and a final zoospore concentration of 500 per ml (=100 per well). Sterility control wells for each preparation contained 200 μl of medium only. Microtiter plates were covered and placed in the dark at 26°C. Growth was evaluated photometrically at 620 nm (42) by measuring optical density in each well at 24-h intervals for 96 h with a microplate photometer (SpectraCount; Packard Instrument Co., Meriden, CT). All values were corrected for optical density at time zero, and experiments were conducted four times in time for each isolate.

Evaluation of zoospore concentration. Growth in half-strength RPMI was evaluated for four zoospore concentrations: 10, 100, 1,000, and 10,000 zoospores per ml. Separate stock zoospore suspensions from each of the three isolates were standardized to 1×10^4 zoospores per ml and were adjusted using a 10-fold serial dilution to the desired zoospore concentrations. Adjusted zoospore suspensions were dispensed in 100-μl aliquots into 21 replicate wells of a 96-well microtiter plate and diluted with 100 μl of half-strength RPMI. Sterility and growth were evaluated as described previously, and experiments were conducted three times in time for each isolate.

Evaluation of antifungal compounds. Technical grade active ingredients (a.i.) of six commercially available fungicides effective against *Phytophthora* spp. were evaluated: azoxystrobin (94.7% a.i.; Syngenta Crop Protection, Greensboro, NC), copper hydroxide (100% a.i.; Chem Service, West Chester, PA), etridiazole (99.5% a.i.; Chem Service), fosetyl-Al (>90% a.i.; Chem Service), metalaxyl (99% a.i.; Chem Service), and propamocarb (99% a.i.; Chem Service). Two broad-spectrum antifungal compounds with limited efficacy against *Phytophthora* spp. also were included: pentachloronitrobenzene (PCNB; 99% a.i.; Chem Service) and pimaricin (95% a.i.; Chem Service). Chemicals were stored in a desiccator at 4°C in the dark to maintain and preserve fungicide activity. Sixty milligrams of each chemical was dissolved in 300 μl of sterile distilled water (copper hydroxide and metalaxyl), 95% ethanol (fosetyl-Al), or dimethylsulfoxide (DMSO; all others), and diluted with sterile distilled water to yield suspensions of 40 mg of a.i. per ml (90% a.i. was used for fosetyl-Al). At the volumes added, ethanol and DMSO had no effect on growth relative to controls. Serial dilutions were made in sterile distilled water to obtain six stock suspensions for each chemical at 10, 5, 1, 0.5, 0.1, and 0.05 mg of a.i. per ml. One hundred microliters of each stock suspension was diluted with 400 μl of half-strength RPMI and dispensed into test wells. Each test well received 100 μl of a chemical concentration and 100 μl of zoospore suspension (1,000 zoospores per ml) in half-strength RPMI. Growth control wells contained 100 μl of half-strength RPMI and 100 μl

of zoospore suspension; treatment control wells contained 100 µl of a chemical concentration and 100 µl of medium without zoospores; sterility controls contained 200 µl of medium only. Final test concentrations were 1,000, 500, 100, 50, 10, and 5 µg of a.i. per ml. For metalaxyl and etridiazole, concentrations of 1.0, 0.5, 0.1, 0.05, 0.01, and 0.005 µg of a.i. per ml also were evaluated. All assays included three replicate wells for each chemical concentration. Microtiter plates were incubated as previously described, and growth was evaluated at 48 h. Percent growth was calculated by dividing the corrected optical density readings of each well by the mean corrected optical density of growth control wells. Each experiment was conducted six times in time per isolate.

Amended agar method. Mycelium-based agar amendment was selected as a control for comparative purposes because it is commonly employed for screening compounds against species of *Phytophthora*. Several 125-ml Erlenmeyer flasks, each containing 45 ml of corn meal agar (CMA; Difco Laboratories), were autoclaved and cooled to approximately 55°C. The CMA was amended with 5 ml of a fungicide stock suspension to produce the same final concentrations as those evaluated in the microbioassay. Ethanol and DMSO had no effect on growth at the concentrations evaluated; control flasks received 5 ml of sterile distilled water. Media were dispensed in 4.5-ml aliquots into disposable petri dishes (60 × 15 mm). Once the media solidified, a 5-mm agar plug from the margin of a 4-day-old V8A colony was placed in the center of each dish. Three replicate dishes were used for each fungicide concentration and unamended control. Cultures were incubated in the dark at 26°C. After 4 days, diameters of individual colonies were measured. Percent growth was calculated by subtracting the diameter of the agar plug (5 mm) from the diameter of each colony and dividing by the mean diameter of the unamended control colonies. These assays were conducted twice in time for each of the three isolates.

Statistical analysis. The regression value (R^2) for the standardization of zoospore concentration was calculated with the regression function in Excel 7.0 (Microsoft Corporation, Redmond, WA). Analyses of medium, zoospore concentration, and effective concentration values for 50% growth reduction (EC_{50}) were performed using StatView, version 5.0.1 (SAS Institute, Cary, NC). Log-transformed growth measurements in medium and zoospore concentration experiments were compared at 24, 48, 72, and 96 h by two-way analysis of variance (ANOVA) with isolate and either concentration or medium as random effects (50). Growth dynamics at each zoospore concentration were assessed by two-way ANOVA with isolate and time as random effects. EC_{50} values predicted by the microtiter and amended agar assays were calculated by fitting a dose-response function to a logistic model (3,8,58). Log-transformed EC_{50} values from the two assays were compared by a separate two-way ANOVA for each fungicide with isolate and assay fixed. The main effects in all variance analyses were compared by Fisher's protected least significant difference tests with $P = 0.05$ (50). Differences in EC_{50} values predicted by the two assays for individual isolates were evaluated with unpaired t tests.

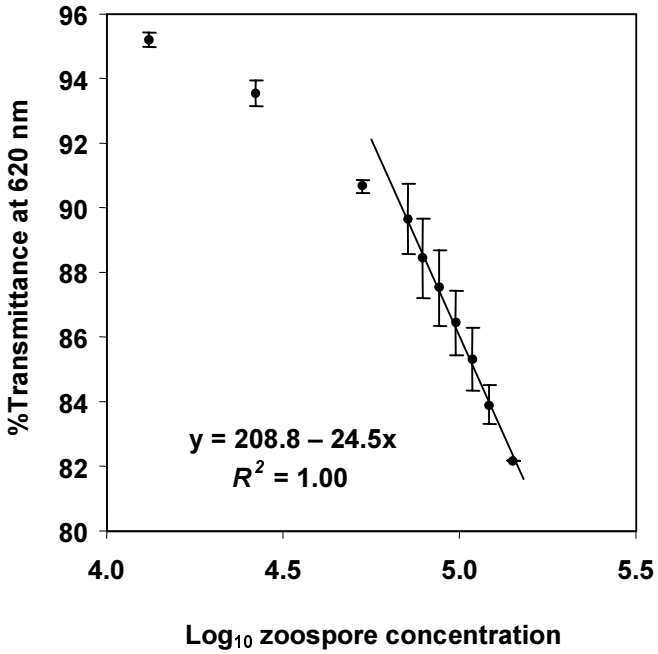

Fig. 1. Regression line for spectrophotometric quantitation of zoospore suspensions of *Phytophthora nicotianae* isolate AF016. Each of three replicate stock suspensions was diluted serially with sterile distilled water to yield 10 concentrations. The resulting suspensions were enumerated with a hemacytometer, and evaluated for percent transmittance at 620 nm. A linear relationship was found between 0.7 and 1.5 × 10^5 zoospores per ml. Data are means from the three replicates; error bars are ±1 standard deviation.

RESULTS

Standardization of zoospore concentrations. To establish a spectrophotometric method for determining zoospore concentration, a regression line for transmittance versus $\log_{10}$ zoospore concentration was plotted for concentrations between 0.15 and 1.5 × 10^5 zoospores per ml (Fig. 1). Percent transmittance was a more sensitive measure of concentration differences than was optical density at the zoospore concentrations evaluated (J. M. Kuhajek, *unpublished data*). A linear relationship was found in the range of 0.7 to 1.5 × 10^5 zoospores per ml or 82 to 90% transmittance (R^2 = 1.00). At concentrations below 0.7 × 10^5 zoospores per ml (i.e., greater than 90% transmittance), transmittance measurements were found to overestimate actual zoospore concentrations. At the highest concentration evaluated, 1.5 × 10^5 zoospores

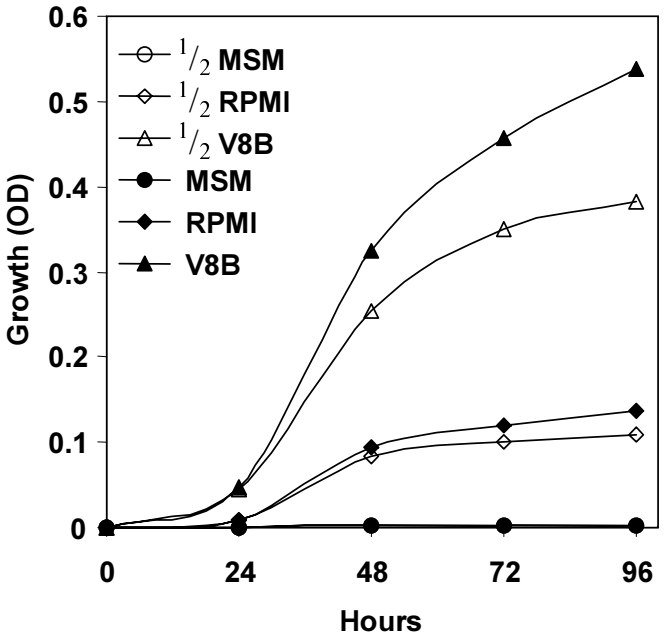

Fig. 2. Growth of *Phytophthora nicotianae* in full- and half-strength preparations of three liquid media at 26°C. A suspension of zoospores (1 × 10^3 zoospores per ml) was added to each medium and growth was monitored at 24-h intervals for 96 h by measuring optical density (OD) at 620 nm. All values are corrected for OD at time zero. Data are pooled means of three isolates in four experiments. Growth curves for full- and half-strength mineral salts medium overlap. Results from statistical analyses are shown is Table 1. Media are as follows: V8B (V8 broth), 1/2 V8B (half-strength V8B), RPMI (Roswell Park Memorial Institute mycological broth), 1/2 RPMI (half-strength RPMI), MSM (mineral salts medium), and 1/2 MSM (half-strength MSM).

per ml, the relationship between transmittance and zoospore number remained linear; however, higher concentrations were undesirable because a large dilution would be required to yield the desired standardized zoospore concentration of 1×10^4 zoospores per ml. Therefore, a concentration of 1.5×10^5 zoospores per ml was considered the upper limit of the useful linear range.

Selection of a suitable synthetic medium. To identify an appropriate medium for the assay, growth of standardized concentrations of zoospores from three isolates of *P. nicotianae* was evaluated in full- and half-strength preparations of two synthetic and one natural medium (Fig. 2). The isolates grew in RPMI and V8B, but not in MSM; growth in both full- and half-strength MSM was nearly undetectable over the entire evaluation period (optical density of ≤0.003; Fig. 2). In the remaining preparations, growth was photometrically quantifiable by 24 h and differed depending on medium (Table 1). Growth at each 24-h interval was superior ($P < 0.01$) in both V8 media compared with the two RPMI media (Fig. 2). Supplementing any of the tested media with additional Ca^{++} (in the form of $CaCl_2$) resulted in suppression of growth (J. M. Kuhajek, *unpublished data*). Growth was similar ($P = 0.55$) in both RPMI and half-strength RPMI throughout the 96 h (Fig. 2). Therefore, both full- and half-strength RPMI were identified as suitable synthetic media for zoospore germination and subsequent mycelium growth of *P. nicotianae*.

Optimum zoospore concentration. The optimum zoospore concentration for initiating growth in half-strength RPMI was determined by testing growth dynamics at four different initial zoospore concentrations (Fig. 3). Growth at the four concentrations was similar at 24 h but differed at 48, 72, and 96 h (Table 2). Growth at 48 h was significantly different at each zoospore concentration tested and was greater at higher concentrations ($P < 0.01$). By 72 h, however, growth at 100, 1,000, and 10,000 zoospores per ml was similar ($P > 0.41$) and greater than growth at 10 zoospores per ml ($P < 0.01$). A lag phase of at least 24 h was observed at each concentration, after which growth increased steadily to 72 h (Fig. 3; $P < 0.01$), except with 10,000 zoospores per ml. At this high concentration, growth reached a plateau between 48 and 72 h ($P = 0.08$) and thus was too rapid for reliable assessment using optical density. By 96 h, growth had leveled off for all concentrations except 10 zoospores per ml. A concentration of 1,000 zoospores per ml provided the most rapid and reliable growth under the bioassay conditions evaluated. At this concentration, photometric growth assessment was ideal at 48 h (Fig. 3).

Comparison of microtiter and amended agar assays. EC_{50} values predicted by the microbioassay and the amended agar assay for each of the eight antifungal compounds were compared by two-way ANOVA (Table 3). EC_{50} values differed significantly between the two assay methods for seven of the compounds; propamocarb was the only compound for which EC_{50} values were similar. However, significant isolate by assay interactions occurred for three of the eight compounds—azoxystrobin, copper hydroxide, and pimaricin. For these compounds, EC_{50} values were compared for each individual isolate and significant differences between the assays occurred for only one of the three isolates. In each case, EC_{50} values were greater using the microbioassay; differences between the assay methods were 53 µg/ml for copper hydroxide, 74 µg/ml for azoxystrobin, and 102 µg/ml for pimaricin. EC_{50} values for three of the remaining four compounds were also greater using the microbioassay; these differences were 104 µg/ml for fosetyl-Al and 1,000+ µg/ml for both etridiazole and metalaxyl. PCNB was the only compound with greater EC_{50} values using the amended agar assay; EC_{50} values for PCNB were greater by 1,061 µg/ml with the amended agar assay compared with the microbioassay.

DISCUSSION

In this investigation, we have developed a standardized microscale bioassay for evaluating the efficacy of antifungal compounds against *P. nicotianae*. Whereas any one bioassay likely will miss some active compounds, the microbioassay provides a reproducible, quantitative procedure for rapidly testing small quantities of sample. The assay is useful for screening both natural and synthetic compounds and mixtures, including natural product extracts (32), and should be useful with other species of *Phytophthora*.

The standardization of methodology is critical to the reproducibility of any quantitative bioassay (22). Lazarovitis (34) demon-

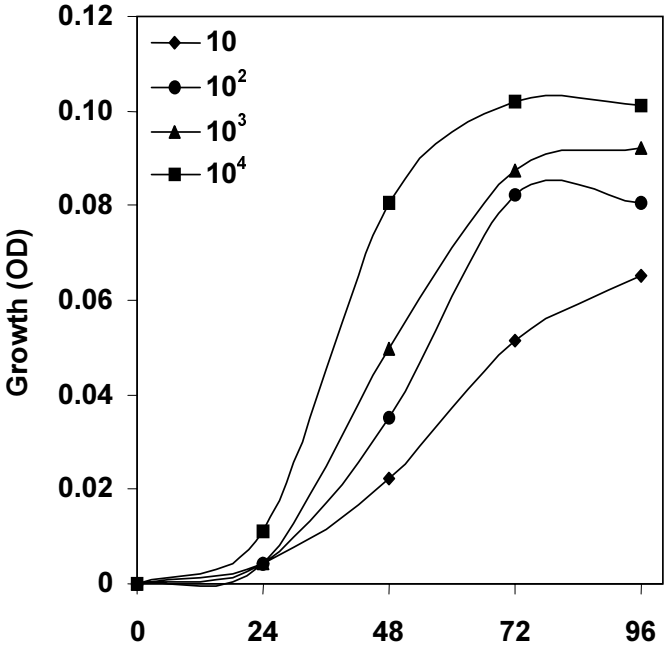

Fig. 3. Growth of *Phytophthora nicotianae* from four initial zoospore concentrations (10, 10^2, 10^3, 10^4 zoospores per ml) at 26°C in half-strength Roswell Park Memorial Institute mycological broth. Growth was monitored by measurement of optical density (OD) at 620 nm at 24-h intervals for 96 h. All values are corrected for OD at time zero. Data are pooled means of three isolates in three experiments. Results from statistical analyses are shown in Table 2.

TABLE 1. *F* statistics and probabilities of greater *F* values (*P*) from two-way analyses of variance of growth at 24-h intervals for three isolates of *Phytophthora nicotianae* in full- and half-strength preparations of two different media[a]

		24 h		48 h		72 h		96 h	
Factor[b]	df[c]	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Isolate	2	0.98	0.43	27.78	<0.01	49.18	<0.01	75.19	<0.01
Medium	3	11.53	<0.01	152.78	<0.01	240.44	<0.01	421.05	<0.01
Isolate–medium	6	2.08	0.08	0.54	0.78	0.51	0.80	0.36	0.90

[a] Data for these analyses are shown in Figure 2.
[b] Data were pooled from four experiments. Isolates did not grow in full- or half-strength mineral salts medium; results from these media were not included in the analyses.
[c] Degrees of freedom.

strated that zoospore concentration significantly influenced the sensitivity of *P. sojae* to fungicide treatment; different concentrations resulted in inconsistent EC_{50} values. The assay reported here incorporates a standardized procedure for preparation of zoospore suspensions, a suitable synthetic growth medium, and an optimized initial concentration of zoospores.

Zoospore suspensions of *P. nicotianae* were measured reliably within a useful concentration range for preparation of initial zoospore suspensions using percent transmittance. The concentration of zoospore suspensions typically is determined with a hemacytometer (7,23,24,46); however, this method of quantitation can produce variable results and can be laborious, especially when working with multiple isolates and replicate subsamples. In contrast, spectrophotometry is rapid and reliable (21,44,57). Because *Phytophthora* spp. do not produce conidia like many fungi, the selection of a suitable propagule for a microbioassay was challenging. A suspension of homogenized mycelium fragments was considered but quickly deemed unsuitable for three reasons. First, a suspension of fragmented mycelium results in a heterogeneous suspension and subsequently a high standard deviation in optical density values (5). Second, members of the genus *Phytophthora* are coenocytic with few cellular divisions in actively growing hyphae; therefore, homogenization resulted in inconsistent viability among propagules (J. M. Kuhajek, *unpublished data*). Third, zoospores are considered the primary infective propagule for *Phytophthora* spp. (17) and thus are a more appropriate assay target than mycelium.

Growth from zoospores was superior in the natural medium, V8B, compared with the two synthetic media evaluated. Similar results were reported by Luo et al. (35). However, an undefined, natural medium is undesirable for sensitive bioassays due to variability in batch-to-batch composition. To ensure maximum assay reproducibility, a completely synthetic, chemically defined medium should be used for sensitive bioassays (38). Growth requirements for most species of *Phytophthora* are relatively simple: calcium, thiamin (0.2 to 1.0 mg/liter), several inorganic salts, and a suitable source of nitrogen and carbohydrates (20). A number of synthetic media have been reported to support growth of *Phytophthora* spp. (20) and many probably are suitable for a microtiter format. For maximum efficiency, a medium with minimal preparation time and few individual ingredients was desired. The most obvious choice was the MSS used for zoospore production amended with a carbohydrate source and a small amount of thiamin. Under the conditions evaluated however, neither full- nor half-strength preparations of this medium supported quantifiable growth of *P. nicotianae*. Alternatively, a commercially available, somewhat more complex medium, RPMI, was selected because it is readily available, simple to prepare, and has been recommended as the standard test medium for several other fungi (38,39,57). In the microbioassay, buffered RPMI supported adequate growth of *P. nicotianae*. Although a 50% dilution of V8B resulted in significantly less growth, no significant difference was observed when RPMI was diluted to half-strength. Because use of a diluted medium significantly reduces the expense of an assay, half-strength RPMI was selected as the medium of choice for the microbioassay.

The optimum initial concentration of zoospores for growth in half-strength RPMI was 1,000 zoospores per ml, which resulted in a final concentration of 500 zoospores per ml in each microtiter well. Concentrations previously employed in zoospore experiments have been similar (7,24) or higher, i.e., 1.5×10^4 zoospores per ml (46). In our experiments, growth was superior at the highest concentration evaluated, 10,000 zoospores per ml. At this concentration, however, logarithmic growth was comparatively brief and explosive. For maximum reproducibility of bioassay results, evaluation must occur during the logarithmic phase of growth; growth during this phase should be moderately rapid and substantial at the time of evaluation. Evaluation of growth using an initial concentration of 1,000 zoospores per ml was possible at 48 h.

EC_{50} values predicted by the microbioassay and the agar amendment assay were similar for only one of the fungicides tested, propamocarb. However, EC_{50} values predicted by the two methods differed by less than 105 μg/ml for four of the compounds—azoxystrobin, copper hydroxide, fosetyl-Al, and pimaricin. When differences occurred, EC_{50} values usually were greater in the microtiter assay. These differences, in part, reflect the superior sensitivity of the microtiter format. The amended agar method measures linear mycelium extension and does not take into account spore germination or colony density. The microbioassay, however, incorporates both zoospore germination and mycelium growth and measures three-dimensional growth of mycelia. EC_{50} values predicted by the microbioassay were consistent with previous reports for all antifungal agents tested except PCNB and pimaricin (7,23,24,29,37,53,55).

PCNB, or quintozene, is a broad-spectrum organochlorine fungicide and often is used in selective media for isolation of *Phytophthora* spp. (20,54). Concentrations of 100 μg/ml typically are used without detriment (20,55). Although effective against many fungus species with chitin cell walls, including many Basidiomycetes and some Ascomycetes, PCNB is virtually inactive against *Phytophthora* spp., which lack chitin (36). Results from the microbioassay ostensibly indicate that PCNB was one of the more active fungicides evaluated. These results are unexpected given the extensive precedence in the literature for the uncompromised isolation and growth of many species of *Phytophthora* in the presence of PCNB (20,54). Because growth was measured indirectly using optical density, the observed activity could be attributable to the combined low aqueous solubility of PCNB and the potential dissolution of the compound in the presence of *P. nicotianae*. In all replicate analyses, EC_{50} values for PCNB were similar to the concentration at which precipitation of this compound was visually apparent. Dissolution of PCNB concurrent with increasing mycelium growth would result in a relatively static optical density reading and thus the false identification of inhibitory activity, as was observed.

Like PCNB, pimaricin, a polyene antibiotic, is routinely used in selective media for the isolation of species of *Phytophthora* (18–20,40,54,55). *Phytophthora* spp. lack endogenous β-hydroxy sterols and thus are resistant to the inhibitory effect of polyenes (31,40). Spore germination, however, is inhibited at concentrations of 100 μg/ml and partial inhibition of germination occurs at lower concentrations (40,55). EC_{50} values predicted for pimaricin

TABLE 2. *F* statistics and probabilities of greater *F* values (*P*) from two-way analyses of variance of growth at 24-h intervals for three isolates of *Phytophthora nicotianae* at four initial zoospore concentrations[a]

		24 h		48 h		72 h		96 h	
Factor[b]	df[c]	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Isolate	2	1.30	0.34	27.25	<0.01	33.28	<0.01	125.96	<0.01
Concentration	3	1.60	0.29	13.62	<0.01	7.24	<0.01	11.57	<0.01
Isolate–concentration	6	1.57	0.20	1.77	0.45	2.51	0.05	1.00	0.45

[a] Data for these analyses are shown in Figure 3.
[b] Data were pooled from three experiments.
[c] Degrees of freedom.

by the microtiter and amended agar assays were similar for two of the three isolates and differed by only 102 µg/ml for the third isolate. Similarity in the results of the two assays presumably reflects the sensitivity of the microbioassay to low levels of zoospore germination and initial growth.

Azoxystrobin is a broad-spectrum fungicide that primarily targets spore germination through inhibition of mitochondrial respiration (27). Matheron and Porchas (37) reported an EC_{50} value of 256 µg/ml for inhibition of zoospore germination of one isolate of *P. nicotianae* from citrus by azoxystrobin; EC_{50} values for azoxystrobin determined by our microbioassay were similar. Matheron and Porchas (37) also reported an EC_{50} value of >3,000 µg/ml when azoxystrobin was tested for inhibition of mycelium growth of *P. nicotianae*. In the amended agar assay in this study, EC_{50} values for azoxystrobin ranged from 56 to 165 µg/ml for inhibition of mycelium growth of three isolates of *P. nicotianae* from ornamental crops. Reasons for the difference between the results of the two studies are not known.

In contrast to azoxystrobin, both metalaxyl and etridiazole are potent inhibitors of mycelium growth. EC_{50} values previously reported for metalaxyl against *P. nicotianae* range from 0.15 to 0.38 µg/ml (10,24,37,51). Chan and Kwee (7) obtained similar values for etridiazole against *P. palmivora* (i.e., 0.001 to 0.21 µg/ml). Higher concentrations were required for 50% inhibition of zoospore germination, i.e., 280 µg/ml for metalaxyl (37) and 10 to 70 µg/ml for etridiazole (7). In this study, results from the agar amendment assay were consistent with these other reports, but all three isolates of *P. nicotianae* tested were insensitive to both compounds in the microbioassay. A probable explanation for the low activity of these fungicides in the microbioassay lies in their mechanisms of action. Metalaxyl inhibits ribosomal RNA synthesis (13). Because intact zoospores contain sufficient ribosomes for germ tube formation, metalaxyl becomes effective only after sufficient growth has occurred (28). Germ tube formation and subsequent mycelium growth of *P. nicotianae* were uninhibited in three separate amended agar trials employing a zoospore suspension rather than mycelium plugs (J. M. Kuhajek, *unpublished data*), and zoospore germination was unaffected by metalaxyl concentrations up to 600 µg/ml (51), indicating that metalaxyl is ineffective against germinating zoospores. Similarly, etridiazole has limited efficacy against germinating spores because it acts through inhibition of mitochondrial respiration by blocking electron transport between cytochromes b and c (45). However, when the etridiazole is present in sublethal concentrations, an alternative electron transport pathway using ubiquinone can be activated, allowing an isolate to appear to be tolerant of high etridiazole concentrations (30).

Sensitivity levels of *P. nicotianae* to copper hydroxide, fosetyl-Al, and propamocarb in the microbioassay generally were consistent with previously published results. Copper compounds, including copper hydroxide, act as enzyme poisons (20) and are highly toxic to *Phytophthora* spp. (29). Copper, in the form of copper oxide, was found to inhibit both mycelium growth and zoospore germination of *P. palmivora* at less than 100 µg/ml (53). In contrast to the general toxicity of copper compounds, fosetyl-Al selectively inhibits mycelium growth through effects on amino acid metabolism (49). Matheron and Porchas (37) found an EC_{50} value of 31 µg/ml for inhibition of mycelium growth of *P. nicotianae* by fosetyl-Al, whereas Farih et al. (23) reported a value of 929 µg/ml and Guest (29) reported an intermediate value of 140 µg/ml. Propamocarb is relatively ineffective against zoospore germination (7,29) but is moderately inhibitory to mycelium growth and severely retards germ tube formation (46). Guest (29) reported 14 to 96% inhibition of mycelium growth by *P. nicotianae* at 100 µg/ml, depending on the growth medium employed.

Based on the combined results for all antifungal compounds evaluated in this study, an EC_{50} value of 200 µg/ml in the microbioassay appears to be an appropriate threshold to identify a compound with potential inhibitory activity against *Phytophthora* spp. and, therefore, worthy of secondary testing. With the exception of metalaxyl and etridiazole, all the fungicides tested with established efficacy against *Phytophthora* spp. exhibited EC_{50} values that were less than 200 µg/ml. As with many in vitro bioassays, however, results obtained using the microbioassay may not always correlate to in vivo efficacy, especially because members of *Phytophthora* have numerous life stages, each with different susceptibility to fungicides (20). The assay does not detect inhibitory activity of compounds that do not inhibit spore germination (e.g., metalaxyl and etridiazole). As with any aqueous-based assay, including agar amendment, analysis of highly hydrophobic compounds (e.g., PCNB) also can be problematic because precipitation can interfere with analysis of growth. Filtration of opacuous suspensions through a membrane filter prior to the addition of zoospores should help to alleviate this problem. Regardless of whether filtration is used, however, results from insoluble compounds are qualitative at best and should not be used in quantitative calculations.

Despite some limitations, the advantages of a microbioassay for *Phytophthora* spp. are numerous. Growth medium and test compound requirements for the microtiter assay are approximately 20-

TABLE 3. Comparison of a new microtiter bioassay (MT) and the standard agar amendment assay (AA) for determining toxicity of antifungal compounds to three isolates of *Phytophthora nicotianae* (AF016, D003, and D119)

Antifungal compound	EC_{50} value (µg/ml)[a]								ANOVA *P* values[c]		
	AF016		D003		D119		Combined[b]				
	AA[d]	MT[e]	AA	MT	AA	MT	AA	MT	Isolate	Assay	Interaction
Azoxystrobin	56	85	165	158	105	179*	…	…	<0.01	0.01	0.01
Copper hydroxide	65	77	58	111*	77	78	…	…	0.52	0.01	0.02
Etridiazole	…	…	…	…	…	…	0.006	>1,000*[f]	<0.01	<0.01	0.10
Fosetyl-Al	…	…	…	…	…	…	93	197*	<0.01	<0.01	0.08
Metalaxyl	…	…	…	…	…	…	0.63	>1,000*	<0.01	<0.01	0.09
PCNB	…	…	…	…	…	…	1,174	113*	<0.01	<0.01	0.16
Pimaricin	403	493	169	164	158	260*	…	…	<0.01	0.01	0.04
Propamocarb	…	…	…	…	…	…	123	124	0.06	0.56	0.08

[a] EC_{50} value is the effective concentration of an antifungal compound that inhibited mycelium growth by 50% relative to a control. * indicates that EC_{50} values predicted by the two assays were significantly different ($P \leq 0.05$) based on unpaired *t* tests.

[b] When isolate–assay interactions were not significant ($P > 0.05$), data for the three isolates were combined.

[c] *P* values from a two-way analysis of variance (ANOVA) for each fungicide with isolate as a random factor and assay fixed; degrees of freedom = 2, 1, and 2 for isolate, assay, and interaction, respectively.

[d] Results were obtained by measuring radial growth of mycelium from an agar plug on chemical-amended agar. Data are the pooled means from two experiments conducted in time.

[e] Results were obtained by measuring optical density in a liquid medium at 620 nm. Data are the pooled means from six experiments conducted in time.

[f] Value indicates that less than 50% inhibition of growth was detected at the highest concentration tested.

fold less than those for traditional methods like the agar amendment assay. Consequently, evaluation of natural products and other samples with limited availability—those available in milligram to microgram quantities—is feasible (32). Furthermore, laboratory space requirements to conduct the microbioassay are greatly reduced, and with the aid of multiwell pipettors, high-throughput screening is possible. For maximum reproducibility, the assay employs a buffered, pH-adjusted, defined medium and standardized methods for preparation of zoospore suspensions. Photometric evaluation provides unbiased, automated three-dimensional analysis of mycelium growth. In addition, the microtiter format provides an opportunity to conduct mechanism-of-action studies for compounds of interest as well as rapid and efficient evaluation of multiple isolates.

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